

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.

THIS PAGE BLANK (USPTO)



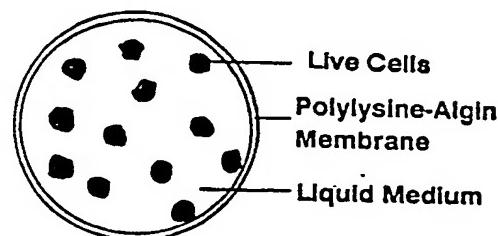
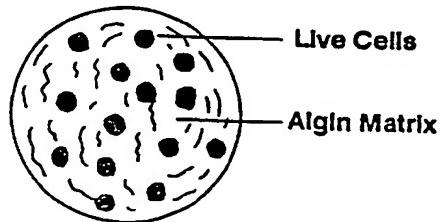
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 9/14, 9/16, 9/52 A61K 9/64, 37/24, C12N 11/04 C12N 11/10, 11/12	A1	(11) International Publication Number: WO 91/01720 (43) International Publication Date: 21 February 1991 (21.02.91)
(21) International Application Number: PCT/US90/04381 (22) International Filing Date: 6 August 1990 (06.08.90)		(74) Agent: GOODMAN, Rosanne; Fulbright & Jaworski, 300 Convent Street, Suite 2200, San Antonio, TX 78205 (US).
(30) Priority data: 389,455 7 August 1989 (07.08.89) US		(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).
(71)(72) Applicants and Inventors: SCHLAMEUS, Herman, Wade [US/US]; 7106 Quail Garden, San Antonio, TX 78250 (US). FOX, William, Casey [US/US]; 7418 Pebblewood, San Antonio, TX 78250 (US). MANGOLD, Donald, Jacob [US/US]; 14711 Bold Venture, San Antonio, TX 78248 (US). TRIPPLETT, Robert, Gill [US/US]; 2906 Bee Cave, San Antonio, TX 78231 (US). HOLT, George, Richard [US/US]; 100 Pin Oak Forest, San Antonio, TX 78232 (US). AUFDEMORTE, Thomas, Bruce [US/US]; 1638 Vista Del Monte, San Antonio, TX 78219 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: COMPOSITION AND METHOD OF PROMOTING HARD TISSUE HEALING

(57) Abstract

Osteoprogenitor cells encapsulated in alginate and alternatively, additionally encapsulated in polylysine and/or agarose promote regeneration of bone at the site of implantation. The present invention provides a composition comprising osteoprogenitor cells embedded or encapsulated in alginate and the use of said microcapsules for the facilitation of bone regeneration.



TWO TYPES OF ARTIFICIAL CELLS

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	PL	Poland
CA	Canada	JP	Japan	RO	Romania
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
DE	Germany	LU	Luxembourg	TD	Chad
DK	Denmark			TC	Togo
				US	United States of America

1

5

-1-

10

COMPOSITION AND METHOD OF PROMOTING
HARD TISSUE HEALING

15 Field of the Invention

The present invention relates generally to the field of hard tissue healing and, more particularly, to the field of biodegradable implantable microcapsules to stimulate the natural process of hard tissue regeneration 20 and bone wound healing.

Background of the Invention

Defects in bone or osseous structures will initiate the process of bone healing. Healing often 25 involves the replacement of injured tissue by connective tissue and leaves a scar. Bone, under optimal conditions, heals by regeneration in which injured tissues are replaced by their own kind and leave no scar. The success of regeneration following injury depends, among other 30 things, on the type of injury, the adequacy of treatment and the systemic health of the patient. Osseous repair involves at least six physiological stages: impact, induction, inflammation, soft callus formation, hard

35

-2-

1 callus formation, remodeling and regeneration.
Heppenstall, Fracture Treatment and Healing, W.B.
Saunders, Philadelphia, 1980, page 35.

5 With inadequate treatment, severe injury and/or metabolic bone disease, fracture healing is significantly retarded. For example, in the case of a metabolic bone disease such as osteoporosis, 40% of patients with decreased bone mass due to osteoporosis showed a markedly impaired fracture repair rate. Only 33% of women in whom 10 significant osteoporosis was present were able to achieve a solid union following femoral neck fractures. In comparison, in 90% of women with physiologically normal bone mass a successful union was achieved. Lane et al., Osteoporosis, Orthopedics clinics North America 15: 711
15 (1984); Arnold, J. Bone Joint Surg. 66A: 847 (1984); Scileppi et al., Surg. Forum 32: 543 (1981).

It is estimated that there are 200,000 hip fractures in osteoporotic women in the United States annually with a 40% mortality rate due to complications of 20 repair of these fractures. As a result, there is a significant need to facilitate fracture repair in these types of patients. In addition, fractures in young accident and trauma victims result in loss of numerous productive days from the work place. For example, it takes an average of six weeks to complete repair even 25 simple bone injuries in healthy individuals.

Bone fractures and bone wound healing following trauma or surgery account for considerable morbidity and mortality. For example, femoral neck fractures in 30 patients under forty may be associated with avascular necrosis in as many as 40% of cases complicated by non-union. Kyle et al., Young Femoral Neck Fractures, Presented at the 52st Annual Meeting of American Acadeym of Orthopedic Surgery, Atlanta, Ga. (1984). Many other examples could be cited of the need for more expeditious
35

-3-

1 methods to facilitate and/or accelerated fracture or hard
tissue defect repair.

5 In addition, recent technological advances have
made the replacement of joints and defective or diseased
hard tissues common surgical procedures.

10 Since the feasibility of the preparation of
artificial cells was first demonstrated in 1957 by Chang
(Chang, T.M.S. (1964) Science:146, 524), numerous
approaches to their production and use have been
evaluated. Artificial cell membranes have been reported
using a variety of synthetic and biological materials to
give the desired membrane properties. A large variety of
15 materials can be enclosed (microencapsulated) in
artificial cells. This includes single and multienzyme
systems, cell extracts, and combined enzyme-adsorbent
systems (Chang, U.S. Patent No. 4,642,120). Biological
cells have been encapsulated to prevent them from being
adversely affected by external factors and immunological
rejection (Chang, Biomedical Applications of Immobilized
20 Enzymes and Proteins (Plenum: New York, 1977) Vols. 1 and
2; Mosbach et al. (1966) Acta Chem. Scan. 20: 2807; Lim et
al. (1980) Science 210:908). More recently the
microencapsulation of living biological cells that can be
maintained in culture has been disclosed (Lim et al.
25 (1980) Science 210: 908; U.S. Patent No. 4,391,909).

U.S. Patent No. 4,663,286 (Tsang et al.)
discloses a process for encapsulating material is
described for forming a capsule utilizing an alginate
polymer with a polyvalent cation.

30 U.S. Patent No. 4,642,120 (Nevo et al.) discloses
the repair of cartilage and bones by employment of a
composition provided in gel form. The gel comprises
certain types of cells. These may be committed embryonal
chondrocytes or any type of mesenchymally-derived cells
35 which may differentiate into chondrocytes, generally as a

-4-

1 consequence of the influence of chondrogenic inducing
factors, in combination with fibrinogen, antiprotease,
thrombin, and other factors. According to U.S. Patent No.
4,642,120, the cells should be of the same species as that
5 to which the composition is transplanted. Incorporation
of extracellular matrix (ECM) of chondrocytes, other
hormones and/or growth factors such as SM (Somatomedin or
IGF-I), FGF (fibroblast growth factor), CGF (cartilage
growth factor), BDGF (bone derived growth factor) or a
10 combination of any of these in the gel is also disclosed.

U.S. Patent No. 4,472,840 (Jefferies) discloses a
method of inducing osseous formation by implanting bone
graft material. Both demineralized bone particles (DBP)
and bone inductive protein have demonstrated the capacity
15 to induce the formation of osseous tissue in animal and
human experiments. Reconstituted collagen conjugate is
known to be highly biocompatible and can be fabricated in
a variety of configurations, especially as a sponge. This
material can be used as a grafting implant in plastic and
20 reconstructive surgery, periodontal bone grafting, and in
endodontic procedures. Structural durability is enhanced
by crosslinking with glutaraldehyde which is also used to
sterilize and disinfect the collagen conjugate prior to
implantation.

25 U.S. Patent No. 4,132,746 (Urry et al.) discloses
a crosslinked insoluble polypentapeptide elastomer capable
of calcification by withdrawing calcium ions from a serum
medium, thus making it useful as a calcifiable matrix for
the formation of an artificial bone structure. The
30 calcifiable material can be treated to make it useful in
artificial vascular wall formation.

U.S. Patent No. 4,609,551 (Caplan et al.)
discusses a material for stimulating growth of cartilage
and bony tissue at anatomical sites. The material
35 consists of a composition with a fibrin or allograft

-5-

1 matrix containing soluble bone protein and fibroblast
cells.

Summary of the Invention

5 In order to facilitate the healing of bone and other hard tissue fractures and defects and facilitate structural implant fixation, a microcapsule has been developed. Specifically, it has been discovered that 10 osteoprogenitor cells can be embedded in or encapsulated by biocompatible materials and nonetheless retain their viability and biological function. The biocompatible encapsulating materials useful in practicing this invention can have different rates of biodegradeability. The biocompatible material may be readily biodegradeable, slowly biodegradeable or relatively resistant to 15 degradation in biological fluids. A readily biodegradeable material is one that is degraded 50% or more within hours to several days by contact with biological fluids. A slowly biodegradeable material will degrade at least 50% when in contact with biological 20 fluids for more than several days up to a week or several weeks. A material resistant to biodegradation is one which retains its integrity for at least several weeks in the presence of biological fluids.

Materials which are readily biodegradeable (bioerodable) include naturally-occurring polymers such as 25 alginates, polylysine, cellulose polymers, e.g., methylcellulose, collagen, gellen gum, casein, chitosan, and the like. Materials which are slowly degradable include some polyesters, and polyanhydrides. 30 Biocompatible materials which are relatively resistant to biodegradation include titanium oxide, hydroxyapatite, biocompatible metal compositions, biocompatible ceramic compositions, and the like. The microcapsules of the present invention can comprise one biodegradeable material 35 or a combination of two or more biodegradeable materials.

-6-

1 In the latter case the microcapsule may contain
biocompatible materials of varying rates of
biodegradeability.

5 A microcapsule comprising one or more
biodegradeable materials can itself be coated or further
encapsulated by a less readily degradable substance in
order to further delay complete release of the
10 encapsulated material. By carefully choosing the
materials used as the initial encapsulating material and
the subsequent coating or encapsulating material, one of
skill in the art may control the rate of release of one or
several encapsulated materials, including the encapsulated
15 osteoprogenitor cells. For instance, in one embodiment,
alginate alone can be used as the sole encapsulating
material. In a second embodiment, biodegradability is
retarded by coating thus-prepared alginate microcapsules
with a polyanionic polymer such as polylysine.

In yet another embodiment a core material
relatively resistant to biodegradation, such as a ceramic
20 material to which another material, e.g., one of the above
mentioned growth factors, has been bound and from which
this other material is slowly released, e.g., from the
surface of the core material, may be encapsulated within a
more readily biodegradeable material which itself contains
25 the same or other treating materials, e.g., the same or
another growth factor, antiviral agent, hormone, in order
to sustain release of one or more of the encapsulated
materials. For instance, a microcapsule comprising woven
titanium mesh mixed with collagen may be also be embedded
30 within the algin microcapsule containing osteoprogenitor
cells. Prosthetic devices formed of the present invention
will facilitate fixation of orthopedic devices or dental
implants by enhancing the bone regeneration at the site of
prosthetic implantation. In another embodiment, fixation
35 of orthopedic implants at the surgical site can be

-7-

1 facilitated by implantation of the composition of the
present invention comprising ceramic hydroxyapatite
adsorbed with bone derived growth factor or any other
material which stimulates the differentiation or growth of
5 osteoprogenitor or cartilage progenitor cells with the
progenitor cells in the formable materials useful for
practicing the present invention. This embodiment allows
a stable and solid support for replacement and/or
reconstruction of defective hard tissues while
10 additionally providing the necessary progenitor cells to
repair and/or replace the defective hard tissue
structures.

As indicated above, microcapsules prepared in
accordance with this invention can additionally contain
15 materials which aid in bone healing or in the prevention
or treatment of complications of trauma. Such additional
materials can include, but are not limited to,
extracellular matrix of chondrocytes (ECM), hormones,
growth factors such as somatomedins, fibroblast growth
20 factor, bone morphogenic protein, platelet derived growth
factor, bone inductive growth factor, osteoinductive
growth factor, cartilage derived growth factor,
prostaglandins, macrophage derived growth factors, bone
derived growth factor, skeletal derived growth factor,
25 epidermal growth factor, transforming growth factor β ,
growth factor, cytokines, and the like, or a combination
of any of these. Such materials may alternatively be
termed herein hard tissue promoting factors. Other agents
which aid in treatment or prevention of the complications
30 of trauma may additionally be included. Examples of such
other agents are, without limitation, antiviral agents,
antibacterial agents and the like. The above agents and
factors may be used alone or in combination in practicing
the present invention. Such materials can be prepared by
35 any method known to those skilled in the art, including

-8-

1 purification from naturally occurring sources and recombinant technology.

5 The microcapsules of this invention, coated or uncoated, e.g., with polylysine, can be further surrounded by a material that can be formed into a hydrogel wafer, such as agar, gelatin, gellan gum or the like, in order to facilitate handling and transfer or implantation of encapsulated material(s) into the site of treatment.

10 The present invention provides compositions and a method to facilitate the healing or regeneration of bone, for instance, at fracture sites. This method comprises implantation or injection of any of the compositions of the present invention into a site or a device in an individual at which bone fixation, reconstruction, 15 regeneration or healing is desired. The osteoprogenitor cells then proliferate and cause the deposition of new bone material at the implantation or injection site.

15 The present invention also provides compositions and a method to facilitate the regeneration and healing of 20 cartilagenous tissues.

It is, therefore, an object of the present invention to provide a composition to augment and/or facilitate the regeneration or healing of bone tissue at fracture sites.

25 A further object of the present invention is to provide a wafer delivery system for encapsulated osteoprogenitor cell-containing compositions of the present invention.

30 Yet another objective of the present invention is to provide viable encapsulated osteoprogenitor cells for implantation and timed-release at bone fracture sites to augment and facilitate healing of the fracture.

35 A still further object of the present invention is to provide a method of stimulating the healing of bone fractures.

-9-

1 These and other objects, as well as the nature,
scope, advantages and utilization of this invention, will
become readily apparent to those skilled in the art from
the following description, the drawings and the appended
5 claims.

Brief Description of the Drawings

Figure 1 shows a schematic of the two capsule types.

10 Figure 2 shows a schematic of the type of apparatus used to form one type of microcapsule.

Detailed Description of the Invention

15 In order to accomplish the above objects and objectives, the present invention provides, in one embodiment, osteoprogenitor cells embedded or encapsulated in an alginate matrix.

20 In one embodiment of the present invention, osteoprogenitor cells have been encapsulated, viability maintained within artificial membranes, and the cells when implanted in an animal model, subsequently proliferate and maintain their capacity to induce osteogenesis.

25 The osteoprogenitor cells useful in carrying out the present invention can be any cells capable of inducing the formation of regenerated bone (or cartilage) (or the deposition of calcium?). Preferably, these cells are autologous bone progenitor cells harvested from the individual in need of such treatment. These cells may be harvested from the site of the injury or from a distant site for transplantation to the injury site. The primary cells may be used directly or may be expanded by passage in cell culture. In another embodiment, the osteoprogenitor cells may be harvested from another individual of the same specie as the individual to be treated. However, the cells may also be selected from the group consisting of cell lines derived from any 30 mesenchymally cells which will differentiate to form

-10-

1 osseous or cartilagenous tissue. In selecting the
osteoprogenitor cells for use in the present invention it
is only important to try to minimize as much as possible
the rejection of the implanted cells for the period
5 necessary to induce the regeneration of new bone.

10 The preferred composition of the present
invention comprises osteoprogenitor cells embedded or
encapsulated in a biodegradeable material. The cells may
either be embedded in a matrix material by being dispersed
within the matrix material itself or by surrounding the
cells with a biodegradeable material. In either case, in
order to decrease the rate of release of the
15 osteoprogenitor cells from the microcapsule, the cells may
be further encapsulated in a nonbiodegradeable material or
a material which has a prolonged integrity in the host
such as polylysine. Preferably, the matrix material is an
alginic acid, such as sodium alginate. The matrix material
may also be selected from the group consisting of gellan
gum, chitosan, or agarose.

20 The method of encapsulating the osteoprogenitor
cells comprises embedding or encapsulating the cells in a
biodegradeable material by any of the techniques known to
those of skill in the art. Preferably the osteoprogenitor
cells are encapsulated by a modification of the method
25 disclosed in U.S. Patent No. 4,391,909, incorporated
herein by reference. Briefly, osteoprogenitor cells were
gently dispersed in a solution of sterile sodium alginate
and pumped through a needle into a collection bath of 1.3%
calcium chloride containing Tween 20. The alginate
30 embedded cells, also termed herein microcapsules, were
harvested, washed with saline and either used directly for
implantation or injection into the treatment site or
further encapsulated to prolong the integrity in the host.

35 In a preferred embodiment, the microcapsules were
formed into wafers to facilitate implantation. These

-11-

1 wafers were preferably composed of agar, such a wafer is
described in Example 4. The wafer can be of any material
that is biocompatible and can be formed into a hydrogel
having characteristics similar to agar such that the
5 handling and placement of the microcapsules at the
treatment site is facilitated.

The method of treating bone fractures of the
present invention comprises implantation of the
osteoprogenitor microcapsules of the present invention
10 into a fracture site of an individual and allowing
sufficient time for the formation of new bone at the
treatment site. The osteoprogenitor microcapsules may be
implanted by surgical procedures known to those of skill
in the art or may be injected into the fracture site
15 utilizing a suitable pharmaceutical carrier. The choice
of such carriers will be obvious to those in the art.

The term "individual" is meant to include any
animal, preferably a mammal, and most preferably a human,
cat, dog, or horse.

20 Artificial cell preparation was carried out in a
sterile environment. All equipment, materials, solutions,
etc. were either sterilized by the appropriate means or
purchased as sterile before use in the process.

Having now generally described the invention, a
25 more complete understanding can be obtained by reference
to the following specific examples. These examples are
provided for purposes of illustration only and are not
intended to be limiting unless otherwise specified.

30

Example 1

PROCEDURE FOR ENCAPSULATION OF OSTEOPROGENITOR CELLS

A. Preparation of osteoprogenitor cells.

Cells were isolated from canine trabecular bone
specimens. The specimens represented material obtained by

-12-

1 biopsy of the iliac crest of four research grade mongrel
dogs numbered as follows: 4452, 4386, 4593, and 4467.
The biopsy specimens from each dog were processed
individually in order to permit autologous implantation of
5 the cellular material at a later date, thereby
circumventing any possible rejection response and
eliminating the need for immune suppression of the host
dogs.

10 The biopsy material was washed multiple times in
Dulbecco's modified Eagle's medium (DMEM) containing
penicillin (1000 units/ml), streptomycin (1000 ug/ml), and
amphotericin-B (0.25 ug/ml) to remove adherent tissue and
debris. The bony trabeculae were then cut into small
15 pieces (1-2 mm²) followed by a second series of washings
to remove hematogenous elements. The resulting clean
pieces of bone were placed in a 100 mm cell culture dish
in the absence of media and incubated at 37°C in an
atmosphere of O₂/CO₂ (95/5 v/v). After 20 minutes, 10
ml DMEM containing 10% newborn calf serum (NCS) was
20 carefully added to the dish without disturbing the bone
fragments. The dishes were returned to the incubator and
left undisturbed for 5 days. Subsequently, the media was
changed every three days to fresh DMEM, 10% NCS. After 23
days of culture, the cells which had migrated from the
25 bone fragments onto the surface of the culture dish were
removed with trypsin/EDTA (0.125%/1 mM).

These cells were placed in a T-75 culture flask
and designated first passage cells. The cells were
passaged two more times to yield third passage cells
30 which, when confluent, were encapsulated as described
below (Runs 1-30A through 1-31B). Examination of aliquots
of the encapsulated cells suspended in DMEM containing the
vital dye trypan blue, indicated that the cells had
retained their viability during the encapsulation.

-13-

1 procedure. The encapsulated cells were maintained in DMEM
containing 10% NCS at 37°C in an atmosphere of O₂/CO₂
(95/5 v/v) for 24-48 hours prior to preparation for
5 implantation into nonunion sites prepared in the radii of
dogs. Viability experiments revealed that the
encapsulated cells could be maintained in this manner for
three days without a decrease in cell number. In fact,
the cell number increased by 70-90% during this time
period.

10 Cells for implantation in nonunion fracture sites
in dogs were harvested and grown in culture as described
above. Osteoprogenitor cells were incubated in an
incubator (37°C) until ready for use in the encapsulation
process.

15 B. Encapsulation of Cells

Cells were encapsulated by a modification of the
method described in U.S. Patent No. 4,391,909 (Lim). Two
types of encapsulated cells were prepared. In one, cells
were encapsulated (or embedded) in an algin matrix. In
20 the second, the process was carried further and the
alginate embedded cells were further encapsulated using
poly-L-lysine/alginate as the capsule membrane. A schematic
of the two capsule types is shown in Figure 1.

The encapsulated cells were prepared as follows
25 and used for implantation into animals to demonstrate the
effect on fracture healing. Cells from several flasks
were combined, placed in a 15-ml sterile culture tube and
rinsed 3 times with sterile 0.9% saline solution. After
30 decanting the saline solution from culture tube, 10 ml of
sterile sodium alginate solution (about 1%) was added.
The alginate used for most of the cell encapsulation was
sterile Macrocarrir* solution obtained from Bellco Glass,
Inc. The cells were gently dispersed and the
cell/alginate solution was transferred to a sterile
35 syringe. The syringe was placed in a sterile pump device

-14-

1 and connected to the encapsulation device with sterile tubing.

5 A sterile collection bath (containing a 1.3% calcium chloride solution with 0.25 ml of 10% Tween 20) was placed under the encapsulation device. The cells were encapsulated in the alginate and collected in the collection bath. After the alginate encapsulated cells remained in the collection bath for 3-5 minutes, they were passed through fine wire screen baskets. The alginate embedded (encapsulated) cells were then rinsed 2 times 10 with 0.9% saline solution and used in this form as the alginate matrix cell preparation.

15 In some situations when it is desirable to provide polylysine encapsulated osteoprogenitor cells, the alginate embedded (encapsulated) cells were rinsed once with a polylysine solution, preferably about 0.2%. The poly-L-lysine used in the encapsulation was obtained from Sigma Chemical Company and had a molecular weight of approximately 38,000. The cells were then incubated in 20 the polylysine solution for 5-7 minutes, rinsed 2 times with 0.9% saline solution, and finally, rinsed once with an approximately 1.5% sodium citrate solution by incubating the encapsulated cells in the sodium citrate solution for 5-7 minutes. The cells were then rinsed 2 25 times with 0.9% saline solution and 3 times with DME (Dulbecco's Modified Eagle's Medium) for 2-3 minutes.

The cells suspended in approximately 40 ml DME were transferred to a sterile T-75 flask and incubated at 37°C until implantation.

30 The results of the encapsulation procedures are shown on Table 1. In the initial runs (1-1A through 1-9D) only placebo capsules were prepared in order to adjust process parameters to produce the desired type of capsule. Matrix materials evaluated during this period 35 included alginates, casein, chondroitin sulfate, and

-15-

1 collagen. In the preferred embodiment, spheres were
 formed using sodium alginate collected in a calcium
 chloride (CaCl₂) bath as shown in Run 1-7A in Table 1. In
5 forming the capsule, air regulation was used to control
 the droplet size. A schematic of the apparatus used is
 shown in Figure 2.

10 Runs following 1-12A were carried out with
 encapsulating live cells unless otherwise stated. The
 encapsulation of osteoprogenitor cells are designated as
 1-30A through 1-31B in Table 1.

15 Histopathologic analysis was performed on
 encapsulated cells maintained in vitro as well as on
 tissues removed at necropsy from animals implanted with
 microencapsulated osteoprogenitor cells for in vivo
 evaluation. This was accomplished in three phases as
 described in Examples 2, 3, and 4, below.

Example 2

In Vitro Cell Analysis

20 Following encapsulation of the cells, in vitro
 studies were conducted to determine osteoprogenitor cell
 viability and define their morphology within artificial
 cell membranes. Histologic sections were prepared and
 stained with hematoxylin and eosin using encapsulated
25 cells in the following combinations:

- K-1 Alginate + U2OS cells (an osteosarcoma cell line)
 - K-2 Polylysine + U2OS cells
 - K-3 Alginate + normal dog cells (animal #4452)
 - 30 K-4 Polylysine + normal dog cells (animal #4452)
 - K-5 Alginate + FL cell tumor (a transformed human
 tumor cell line capable of bone formation)
- U2OS cells encapsulated in alginate appeared as
 small nests or colonies numbering approximately 2-15
35 cells, each with an average of approximately 10 cells per

-16-

1 group. The cells had basophilic staining nuclei which
were round and regular with prominent nucleoli noted at
random. Cell cytoplasm was moderately eosinophilic and
cell boundaries were relatively distinct. The algin
5 matrix was amorphous and slightly basophilic but obviously
degrading as a consequence of the histologic processing
procedure necessary to produce the sections.

U2OS cells encapsulated in polylysine also
appeared as clusters with morphology not significantly
10 different from that described above, however, the
artificial polylysine membranes were histologically
distinct as slightly basophilic undulating cuticular
surfaces enclosing cell nests. The undulation was
interpreted as an artifact of dehydration, again necessary
15 for processing.

Normal dog cells when encapsulated in alginate,
appeared as isolated groups, usually of 2-3 cells.
Morphologically the cells had the characteristics of
osteoblasts with eccentrically located round nuclei and
20 relatively conspicuous eosinophilic cytoplasm. In some
cells there was evidence of a perinuclear eosinophilic
condensation typical of osteoblasts. Again, the alginate
membranes appeared to be degrading as a result of the
histological preparation.

25 Normal osteoprogenitor dog cells encapsulated in
polylysine showed similar morphology to those encapsulated
in alginate alone. Again, polylysine membranes were
distinct as described with the U2OS cells above.

Alginate embedded FL cells also showed isolated
30 cells or groups of 2-4 cells with round, eccentrically
located nuclei, occasional prominent nucleoli and
eosinophilic cytoplasm, but differing from U2OS cell lines
in that clusters were in general smaller and less numerous
within the artificial membranes.

-17-

1 In summary, all artificial cell preparations
contained viable cells with morphology varying as to the
derivation of the particular cell type indicating that no
5 deleterious effects resulted from the encapsulation
process.

10 Alginate cell membranes degraded during
histological processing and thus were not visible in
subsequent sections produced from animal studies.
Polylysine membranes were more distinct and durable and
15 remained visible at least in early phases of the animal
studies. The interpretation of the in vivo data shown in
the Examples below was made in accordance with these
observations .

20 These studies demonstrate that cells may be
encapsulated, their viability maintained, and sections
prepared for histologic analysis. Intact cells were noted
within the confines of the artificial membranes and, as a
consequence, these formulations rendered viable cells for
implantation studies.

Example 3

In Vivo Studies of Encapsulated Cell Lines Implanted in Nude Mice

25 Cell viability following encapsulation was
evaluated in vivo using FL cells, a transformed line of
human amnion cells capable of tumor formation in the nude
mouse. The rationale for these experiments follows.
Cells encapsulated in alginate and implanted beneath the
30 skin of the nude mouse formed tumors as rapidly as
nonencapsulated cells injected subcutaneously, since the
alginate is rapidly dissolved in vivo. Formation of
tumors by cells encapsulated in polylysine was delayed,
since polylysine is not readily dissolved in the host and
cells first have to multiply within the capsules in
35 sufficient mass to burst them.

-18-

1 Encapsulated FL cells (Runs 1-15B, 1-16B, 1-36A,
and 1-36B) were maintained overnight at 37°C in an
atmosphere of O₂/CO₂(95/5 v/v). The following morning 0.5
ml alginate or polylysine encapsulated cells were
5 surgically implanted beneath the skin of 3-week old nude
mice of the nu/nu strain (Harlan). The mice were
sacrificed at 16 and 32 days after implantation for gross
and histological evaluation of tumor formation.

10 FL cell lines encapsulated in alginate and
implanted for a period of 16 days, demonstrated at
necropsy, viable cells with histologic features remarkably
similar to those described in the in vitro experiments
with the exceptions that the cell clusters were now much
larger, often forming confluent nests in excess of several
15 hundred cells.

20 Alginate membranes, as expected, were not visible
but the general outlines of the artificial cells were
present in some areas, perhaps attributable to
fibrocollagenous connective tissue proliferating in
proximity with the artificial cell membranes. In other
areas, FL cells had grown into confluent nests with the
subcutaneous tissue and muscles, violating and disrupting
the boundaries of the artificial cell membranes. In these
areas of host tumor interface, conspicuous bone and
25 osteoid production was noted.

30 FL cells encapsulated in polylysine and implanted
for 16 days again showed large viable cell clusters with
morphologic features as described with the exception that
the cell membranes of polylysine remained intact. Most
cell groups within the membranes had grown to confluence.
No evidence of cell penetration into adjacent tissues, as
was noted above, was apparent. No bone or osteoid
production was visible.

35 FL cell lines encapsulated in alginate/polylysine
harvested 32 days after implantation showed a large bulk

-19-

1 of tumor (larger than 2.0 X 1.0 cm) with FL cell line
morphology. It was composed of confluent nests and sheets
of cells proliferating in no discernable pattern with a
few groups similar to those described above still
5 present. Most membrane material apparently had been
resorbed and was inconspicuous. There was overt invasion
of host tissue by the FL cell lines with conspicuous bone
and osteoid production.

10 Thus, the above results have demonstrated the
viability of encapsulated cells and further that this
viability could be maintained throughout the implantation
or injection procedure with the encapsulated cells
subsequently proliferating within artificial membranes,
rupturing the membranes and invading into host tissues.
15

15 Additionally, the above results demonstrate that
cell lines induced bone production, evidence of the
maintenance of cell capacity to exhibit their normal
function following the encapsulation process. Alginate
and polylysine microcapsules apparently degrade at
20 different rates, since discernable differences between
polylysine and alginate encapsulated cells were noted at
16 days with alginate tending to degrade earlier than
polylysine.

25 In order to determine if artificially
encapsulated cells would survive in vivo, 7 nude mice were
injected with encapsulated cells formulated in varying
matrices. If injection of encapsulated cells is delayed,
viability is significantly suppressed. Vital cells
encapsulated in polylysine and alginate membranes could be
30 observed 24 days following injection. Surviving cells
which had been injected alone or in a carrageenan matrix
were not detectable at 24 days.

-20-

1 Example 4

In vivo Studies of Treatment of Fracture Non-unions
Produced in Dogs.

Fracture nonunions were experimentally induced in
5 11 research grade dogs. The nonunions were performed by
surgically removing a 3 mm disc of cortical and cancellous
bone from the mid-radius. Dogs were then allowed to
resume normal weight bearing activities, and after 12
weeks, stable fracture nonunions were produced. The dogs
10 were then divided into groups consisting of controls
receiving only matrix material with no osteoprogenitor
cells and four animals receiving osteoprogenitor cells
formatted in varying ways. Each dog received cells which
had been harvested at the time of the initial surgery and
15 maintained in tissue culture as described in Example 1
above.

In order to facilitate handling during the
implantation procedures and to insure retention at the
nonunion site, the encapsulated cells were prepared in a
20 gel of low melt agarose (Sigma TYPE VII). A "doughnut"
prepared with 3 ml of 4% agarose was formed in a 28 mm
diameter culture dish with a 12 mm diameter post in the
center. After the agarose had gelled, the centerpost was
removed. A suspension was prepared from 3 ml encapsulated
25 cells and 3ml 2% agarose. The hole in the center of the
4% agarose doughnut was filled with 1.5 ml of this
suspension. After the central portion had gelled, the
entire doughnut was transferred to a cell culture dish,
covered with DMLM, and returned to the incubator. The
doughnuts were implanted into the nonunion defects within
30 15-18 hours. The outer rim of the doughnut was
substantial enough to permit gentle handling with
forceps. The central core was rigid enough to hold the
encapsulated cells at the implant site, while still
allowing for diffusion of wound and tissue fluid to the
35

-21-

1 cells. These discs were then implanted following
excision of the fibrous nonunion material and the radii
splinted with a 4-hole stainless steel splint. The dogs
then resumed weight bearing activity for an additional 12
5 weeks at which time the animal was sacrificed and material
taken for detailed histologic evaluation.

10 The two dogs receiving only polylysine matrix
material showed a persistent nonunion defect occupying
approximately 8.5-11% of the original nonunion defect
volume on histomorphometric analysis. The trabecular bone
volume in these areas was calculated at 6.5 and 24.75%
respectively with 46.9 and 18.9% fibrous connective tissue
intermixed as well as a small amount of fibrocartilage.
In addition, a significant quantity of polylysine matrix,
15 visible as irregularly shaped refractile material was
noted throughout the defect. There was a modest
multinucleate foreign body giant cell response to this
material as well as minimal chronic inflammatory cell
infiltration. The histologic features from the two
20 control dog studies were identical to six control dogs
from previous studies involving the encapsulation and
implantation of bone inductive proteins in nonunion
fractures.

25 When autologous osteoprogenitor cells were
encapsulated in an artificial matrix of alginate and
implanted in a dog nonunion, histologic examination showed
a dramatic and complete healing of the fracture nonunion.
This was apparent on histomorphometric analysis with 100%
of the original defect being filled with new bone. The
30 trabecular bone volume in this area was 55% with no
interposed fibrous connective tissue. Relatively normal
cancellous space was present instead. This was in
dramatic contrast to the controls and other test animals
receiving inductive proteins. Also apparent were isolated
35 small cell clusters and groups of cells with round,

-22-

- 1 elliptically located nuclei and relatively distinct cytoplasmic membranes with slight eosinophilia to the cytoplasm. These were identical to cell clusters noted in the in vitro and nude mouse in vivo experiments. These
5 cells could be observed within the cancellous space and at times in intimate adaptation with an acellular eosinophilic homogeneous material consistent with osteoid.
- When autologous osteoprogenitor cells were encapsulated in a polylysine matrix and implanted in a dog nonunion, histologic examination 3 weeks demonstrated evidence of degrading artificial cell membranes consistent with polylysine and a few artificial cell nests as described above in the in vitro and in vivo nude mouse studies, as well as the dog previously described.
- 10 15 Throughout the nonunion site there was evidence of brisk osteoblastic activity with production of homogeneous, eosinophilic acellular osteoid as noted in the previous dog. The histologic features demonstrated healing at a significantly advanced stage compared with that anticipated for control animals from previous nonunion experiments. The two remaining dogs each received polylysine encapsulated cells or alginate encapsulated cells. Both dogs were carried to 13 weeks. The polylysine cells showed some evidence of osteoid
20 25 production and remnants of artificial cells, but no significant fill of the nonunion defect. The same was true of the last dog receiving alginate encapsulated cells.
- The results of the implantation of osteoprogenitor cells encapsulated in alginate (with or
30 without poly-L-lysine) demonstrated that the method of the present invention causes complete healing of the fibrous nonunion, the healed fracture being composed of mature bone with lamellar characteristics and evidence that remodeling of the fracture site into a functional state
35 had occurred. This Example conclusively demonstrates that

-23-

1 osteoprogenitor cells may be encapsulated in artificial
membranes, their viability maintained, and these cells
subsequently implanted in living subjects (mice and
dogs). The cells subsequently proliferate out of the
5 artificial confines to produce osteoid and new bone which
contributes to the healing process.

10 Although all dogs receiving encapsulated
osteoprogenitor cells did not demonstrate the same amount
of nonunion fracture healing, this result may relate to a
number of complex interrelated factors. These include the
kinetics of artificial cell membrane degradation, cell
release from artificial membranes, proliferative
capabilities of individual autologous cells, differences
inherent in healing capacity of each animal, or
15 combinations of these.

In addition, bone inductive factors may be
necessary in the artificial membranes to completely signal
encapsulated cell populations to begin proliferation
within the unfavorable environment of a healing wound.
20 Some of these variables may be overcome by inclusion of
bone cell differentiation factors within the microcapsule
at the time of encapsulation.

Example 5

Device Fixation Enhancement in Primates

25 In order to improve the fixation of titanium bone
implants, in vivo studies of implantation of
microencapsules containing osteoprogenitor cells and bone
inductive factors were performed. Osteoprogenitor cells
were encapsulated in either alginate or alginate coated
30 with polylysine as described in Example 1. Additionally,
microcapsules were prepared as in Example 1 however, bone
inductive growth factor was also included in the
microcapsules. The microcapsules were implanted within
the internal aspects of a titanium bone implant in six
35 sites in each of two baboons (animal no. 713 and 609) to

-24-

- 1 determine whether bone growth into titanium prosthetic implants could be enhanced, facilitating of the fixation of the prosthesis in the baboon tibia.
- 5 One of six titanium implant sites was used as a control and received no microcapsule material. Each of of the remaining five titanium implant sites in baboon tibia received the same microencapsulation composition. At one week intervals for six weeks, tissue within the internal aspects of one of the six titanium implant sites in each 10 baboon was retrieved for histologic evaluation. The status of the encapsulated material and the quantity of bone in the internal aspects of the titanium implant was determined. Histologic analysis of tissue within the implant at the site of microcapsule implantation was 15 carried out weekly for six weeks. The encapsulating materials were highly biocompatible and did not elicit a giant cell foreign body response. The amount of encapsulating material present in histologic sections decreased as the treatment period progressed, indicating 20 that the implanted microcapsules were biodegraded at the site of the titanium prosthetic implant. Gross histological examination revealed bone regeneration in all titanium prosthetic implant sites which received the microcapsule composition of the present invention.
- 25 Further analysis may reveal quantitative or qualitative differences in the regenerating hard tissue due to the presence of the bone inductive growth factor.
- The invention now being fully described, it will be apparent to one of ordinary skill in the art that many 30 changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.
- WHAT IS CLAIMED AS NEW AND IS DESIRED TO BE
35 COVERED UNDER LETTERS PATENT IS:

-25-

1 CLAIMS

1. A composition comprising osteoprogenitor cells encapsulated in a biocompatible microcapsule.
- 5 2. The composition of claim 1, wherein said microcapsule comprises a biodegradeable polymer.
- 10 3. The composition of claim 2, wherein said biodegradeable polymer is selected from the group consisting of alginate, polylysine, methylcellulose, collagen, gellen gum, casein and chitosan.
- 15 4. The composition of claim 3 wherein said polymer is alginate.
5. The composition of claim 3 wherein said polymer is polylysine.
- 15 6. The composition of claim 1 wherein said microcapsule comprises alginate and polylysine.
7. The composition of claim 1 wherein said microcapsule comprises alginate coated with polylysine.
- 20 8. The composition of claim 1 further comprising a material selected from the group consisting of extracellular matrix of chondrocytes (ECM), a hormone, a growth factor, an antiviral agent, and an antibacterial agent.
- 25 9. The composition of claim 8 wherein said growth factor is selected from the group consisting of somatomedin, fibroblast growth factor, epidermal growth factor and bone derived growth factor.
- 30 10. The composition of claim 1 contained in a hydrogel wafer.
11. The composition of claim 10 wherein said hydrogel wafer comprises a material selected from the group consisting of agar, gelatin, gellan gum and agarose.
- 35 12. A method for promoting bone regeneration, comprising administration of the composition of any one of claims 1 - 11, inclusive, to an individual in need of said treatment.

-26-

1 13. The method of claim 12 wherein said individual is a mammal.

5 14. The method of claim 13 wherein said mammal is selected from the group consisting of a human, a dog, a cat, and a horse.

10 15. The method of claim 14 where in said mammal is a human.

10 16. A composition comprising a material resistant to degradation in biological fluids, wherein said resistant material is selected from the group consisting of titanium oxide, hydroxyapatite, biocompatible metal compositions and biocompatible ceramic compositions; hard tissue promoting factors bound to the resistant material; and contained within a readily degradable polymer.

15 17. The composition of claim 16 additionally comprising unbound hard tissue promoting factors.

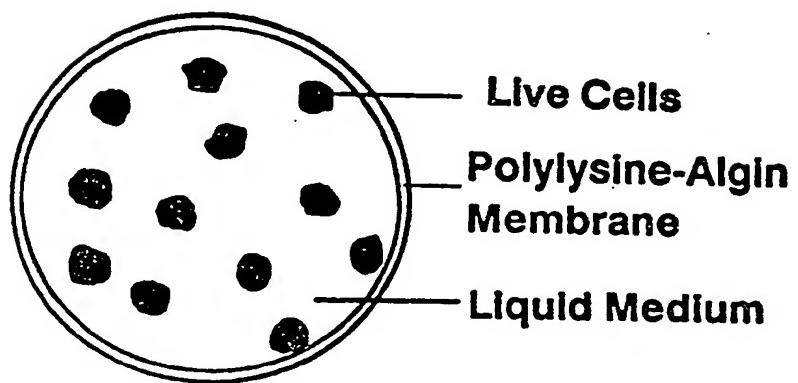
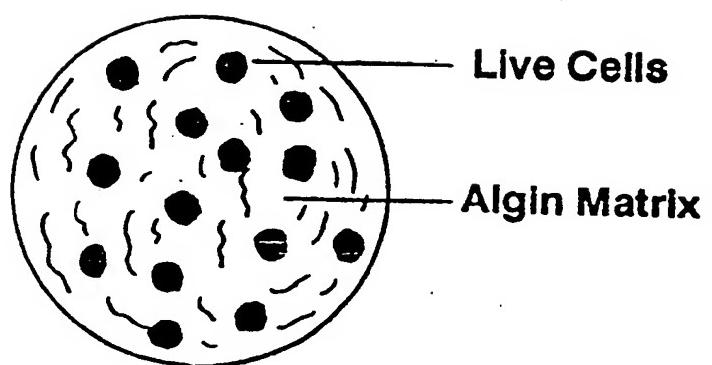
20

25

30

35

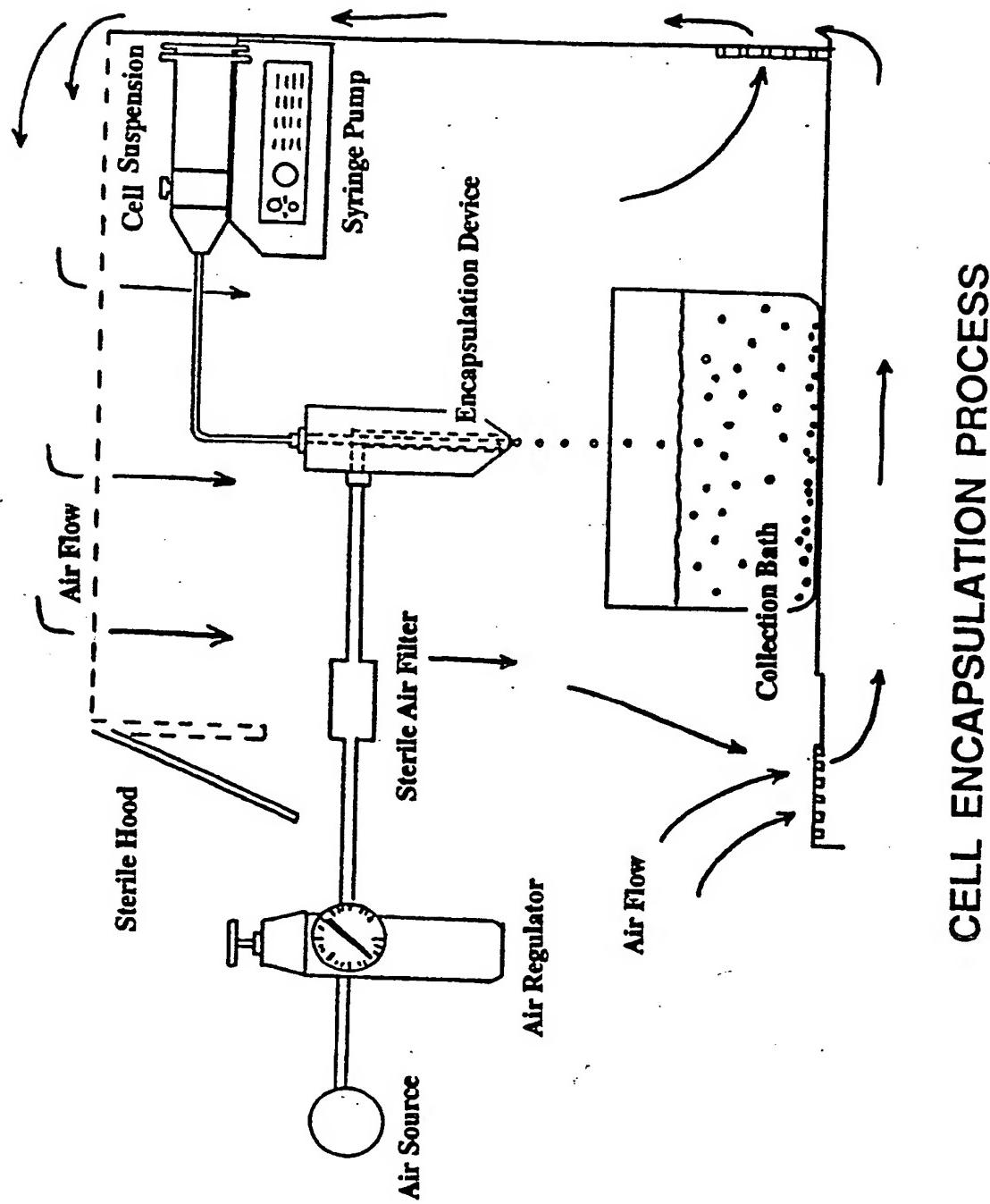
1/2



TWO TYPES OF ARTIFICIAL CELLS

Figure 1

2/2



CELL ENCAPSULATION PROCESS

Figure 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/04381

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶	
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 9/14, 9/16, 9/52, 9/64, 37/24; C12N 11/04, 11/10, 11/12 U.S. CL. 424/484, 485, 488, 491,; 435/178, 182; 530/399	
II. FIELDS SEARCHED	

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
U.S.	424/484, 485, 488, 491; 435/178, 179, 182 530/399
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸	

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A, P	US, A, 4,904,259 (ITAY) 27 FEBRUARY 1990 See Abstract.	1, 2, 12-15
Y	US, A, 4,642,120 (NEVO) 10 FEBRUARY 1987 See Abstract and column 4, lines 35-36.	1, 2, 8, 9, 12-15
Y	US, A, 4,609,551 (CAPLAN) 02 SEPTEMBER 1986 See Abstract, column 2, lines 1-2, 17-22, 54 and column 3, lines 34-48.	1-3, 8, 12-15
Y	US, A, 4,647,536 (MOSBACH) 03 MARCH 1987 See Abstract and column 1, lines 10-15.	1, 2, 3
Y	US, A, 4,663,286 (TSANG) 05 MAY 1987 See Abstract, column 1, lines 5-7 and column 3, lines 4-38.	1-7
Y	US, A, 4,391,909 (LIM) 05 JULY 1983 See Abstract, column 4, lines 9-16, column 9, and Example 1.	1-7
Y	US, A, 4,673,566 (GOOSEN) 16 JUNE 1987 See Abstract, column 8 and Example 1.	1-7, 10, 11

- Special categories of cited documents: ¹⁰
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATE	
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 OCTOBER 1990	09 JAN 1991
International Searching Authority ISA/US	Signature of Authorized Officer S. Kishore Gollamudi S. Kishore

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,798,786 (TICE) 17 JANUARY 1989 See Abstract, column 10 and claim 3.	1-3, 11-15
Y	US, A, 4,620,327 (CAPLAN) 04 NOVEMBER 1986 See Abstract, column 2, lines 4-54 and column 3, lines 27-34.	16 & 17
Y	US, A, 4,610,692 (EITENMULLER) 09 SEPTEMBER 1986; See Abstract.	16 & 17
Y	US, A, 4,595,713 (ST. JOHN) 17 JUNE 1986 See Abstract, and column 6, lines 23-41.	16 & 17
Y	US, A, 4,888,366 (CHU) 19 DECEMBER 1989 See Abstract, column 2, lines 54-64 and column 7, lines 7-65.	16 & 17

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____ because they relate to subject matter ^{is not} required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ^{is}, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-15: A composition comprising osteoprogenitor cells encapsulated in a microcapsule.
- II. Claims 16-17: A composition containing no osteoprogenitor cells.
(See attachment)

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

Con't. from PCT/ISA/210 supplemental sheet (2).

The international application lacks unity of invention under PCT Rule 13 because of the following reason:

Inventions I and II are independent and distinct in that the composition in invention I contains osteoprogenitor cells whereas the composition in invention II does not require the presence of these cells, but contain additional factors.